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Abstract: BACKGROUND: Chronic norovirus infection is an emerging challenge in the immunocompromised host, in whom it may be asymptomatic or present as chronic diarrhea. The mechanisms of diarrhea in chronic norovirus infection are not well understood, but in analogy to Gardia lamblia and rotavirus infections, secondary lactose maldigestion (LM) might be implicated. METHODS: Adult renal transplant recipients (RTRs) who had symptomatic chronic norovirus infection with diarrhea were asked to participate in this prospective parallel cohort study. RTRs with otherwise unexplainable chronic diarrhea but absent infection served as control group. In both groups, a lactose hydrogen breath test (LHBT) and a lactose tolerance test (LTT) were performed after exclusion of primary LM by a negative lactase gene test. RESULTS: Of approximately 800 patients in the cohort of RTRs at our institution, 15 subjects were included in the present study. Of these, 7 had chronic symptomatic norovirus infection with diarrhea (noro group) and 8 had diarrhea in the absence of norovirus (control group). LHBT and LTT were positive in all 7 patients (100%) in the noro group, whereas only 1 of 8 patients (12.5%) in the control group had a positive test. Thus, secondary LM was highly prevalent in the noro compared to the control group with an odds ratio of 75.0 (95% CI 2.6, 2153, p=0.01). CONCLUSIONS: This is the first report showing a positive association of chronic norovirus infection and secondary LM. Further studies with larger patient numbers and longer follow-up are needed to test a causative relationship between both entities.

DOI: https://doi.org/10.1097/TP.0000000000001376

Transplantation Publish Ahead of print
DOI: 10.1097/TP.0000000000001376

Chronic norovirus infection as a risk factor for secondary lactose maldigestion in renal transplant recipients: a prospective parallel cohort pilot study

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Trial registration: This study is registered at ClinicalTrials.gov (identifier: NCT01840891)

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Authors' contributions

Study design (TF, RPW, DF), data collection (RP, MB), data analysis (MB, BM, DF), drafting of the manuscript (MB, BM, TF, RPW, DF). Approval of the final version of the manuscript (all authors).

Disclosures

The authors declare no conflicts of interest

Funding

There is no funding source of this study

ABBREVIATIONS PAGE

CMV, cytomegalovirus

IBS, irritable bowel syndrome

IQR, interquartile range

LHBT, lactose hydrogen breath test

LTT, lactose tolerance test

LCT, lactase

LI, lactose intolerance

LM, lactose maldigestion

PCR, polymerase chain reaction

RTR, renal transplant recipient

PCR, polymerase chain reaction

SIBO, small intestine bacterial overgrowth

ABSTRACT

Background: Chronic norovirus infection is an emerging challenge in the immunocompromised host, in whom it may be asymptomatic or present as chronic diarrhea. The mechanisms of diarrhea in chronic norovirus infection are not well understood, but in analogy to Gardia lamblia and rotavirus infections, secondary lactose maldigestion (LM) might be implicated.

Methods: Adult renal transplant recipients (RTRs) who had symptomatic chronic norovirus infection with diarrhea were asked to participate in this prospective parallel cohort study. RTRs with otherwise unexplainable chronic diarrhea but absent infection served as control group. In both groups, a lactose hydrogen breath test (LHBT) and a lactose tolerance test (LTT) were performed after exclusion of primary LM by a negative lactase gene test.

Results: Of approximately 800 patients in the cohort of RTRs at our institution, 15 subjects were included in the present study. Of these, 7 had chronic symptomatic norovirus infection with diarrhea (noro group) and 8 had diarrhea in the absence of norovirus (control group). LHBT and LTT were positive in all 7 patients (100%) in the noro group, whereas only 1 of 8 patients (12.5%) in the control group had a positive test. Thus, secondary LM was highly prevalent in the noro compared to the control group with an odds ratio of 75.0 (95% CI 2.6, 2153, p=0.01).

Conclusions: This is the first report showing a positive association of chronic norovirus infection and secondary LM. Further studies with larger patient numbers and longer follow-up are needed to test a causative relationship between both entities.

Key Words: Chronic norovirus infection, chronic diarrhea, renal transplant recipients, lactose maldigestion

Introduction

Norovirus, a single-stranded RNA virus of the Caliciviridae family, is a human enteric pathogen that is 1 of the leading causes of acute gastroenteritis, presenting as self-limited disease of short duration in immunocompetent subjects.[1-3] However, chronic norovirus infection is an emerging challenge in the immunocompromised host such as leukemia patients or solid organ transplant recipients, in whom the virus may persist and present as chronic diarrhea and diffuse abdominal discomfort, and may even be associated with kidney transplant dysfunction.[4-8] Norovirus accounts for 17-26% of severe posttransplant diarrhea in renal transplant recipients.[5,6,9] Norovirus related diarrhea is associated with the greatest weight loss compared to other causes of diarrhea.[5,9] Histologically, signs of chronic intestinal inflammation are present.[4,5] Until now, the mechanisms of diarrhea in case of chronic norovirus infection are not well understood, and treatment options are limited.

Lactose is a disaccharide and a frequent constituent of a typical Western-type diet. Lactose maldigestion (LM) refers to inefficient cleavage of lactose in the small intestine, resulting in lactose malabsorption and fermentation of lactose by the colonic microbiota. In contrast, lactose intolerance (LI) is defined as the development of symptoms after lactose challenge in individuals with LM.[10] LM is a frequent condition, affecting more than 50% of all individuals worldwide and should be regarded a variant of human intestinal physiology.[11] Primary LM is typically associated with the CC polymorphism of the -13910 locus of the lactase (LCT) gene.[12] In contrast, secondary LM can develop in many intestinal inflammatory conditions; however, which specific conditions will lead to LM as well as mechanistic aspects, have not been sufficiently clarified.

In a prospective study in children with acute gastroenteritis, a significant proportion was found to have LM, which was most commonly associated with rotavirus infection.[13] Secondary LM has also been reported in patients with Giardia lamblia infections, and the latter were shown to alter the cellular glycocalyx resulting in alterations of brush border disaccharidase enzymes.[14,15] In line with these findings we suspect a similar mechanism in symptomatic patients with chronic norovirus infections. The objective of this study was therefore to determine the prevalence of secondary LM in patients with chronic norovirus infection.

Materials and Methods

Subjects

Between July 2013 and March 2015 all adult renal transplant recipients (RTRs) at the University Hospital Zürich who had symptomatic chronic norovirus infection with diarrhea were asked to participate in this prospective parallel cohort study. According to the WHO-approved definition of diarrhea we chose the cut-off of 3 or more bowel movements per day for more than 4 weeks as indicative of chronic diarrhea. Chronic norovirus infection was proven by positive polymerase chain reaction (PCR) analysis of recent stool samples, whereas chronic virus shedding was defined as more than 2 PCR positive samples at an interval of at least 1 month. Concomitant viral (ie cytomegalovirus), bacterial (ie Salmonella spp., Campylobacter spp., Shigella spp., and C.difficile) and parasitic (Gardia lamblia, Microspora spp., and Cryptospora spp.) intestinal infections were excluded by negative stool PCR analyses, stool cultures, and direct microscopic stool examinations, respectively. Furthermore, CMV viremia was excluded by PCR technique. Main exclusion criterion for the present study was a concomitant intestinal infection (other than norovirus), and primary LM which was previously excluded by absence of

the CC genotype of the DNA variant -13910 T/C upstream in the LCT gene. Subjects with a proven galactosemia or those requiring a low galactose diet were also excluded. RTRs with otherwise unexplainable chronic diarrhea but absent norovirus or another intestinal infection, and negative LCT gene test served as control group. In both groups, a lactose hydrogen breath test (LHBT) and a lactose tolerance test (LTT) were performed in all eligible RTRs (Figure 1). LM was diagnosed with a positive LHBT and/or a positive LTT.

Written informed consent was obtained from all patients included in the study. The study was approved by the local Ethics committee (KEK-ZH 2012-0473) and is registered at ClinicalTrials.gov (identifier: NCT01840891).

Lactose H_2 breath test (LHBT)

The LHBT was performed according to the Rome consensus conference.[16] After an overnight fast of at least 12 hours, a basal breath sample was collected. No individual showed a baseline hydrogen (H_2) level above 20 ppm (not shown). RTRs were allowed to drink water and follow their usual medication regimen during the entire examination. After collecting the baseline sample, RTRs were given 25 g of lactose dissolved in 250 ml of water to drink. Orange flavored lactose powder (or milk powder) was provided with the AlveoSampler™ Lactose Kit (Quintron Instrument Co., Milwaukee, WI, USA). Samples of end expiratory breath were then collected at 30, 60, 90 and 120 minutes after the oral lactose load to measure the concentration of H_2, which was considered significantly increased and indicative of LM when exceeding 20 ppm.[16,17] During the test, RTRs were allowed to engage in normal activities, but were kept fasting except for water consumption which was permitted throughout the examination. The test was performed in a well-ventilated room free of fresh painted walls or objects and with no

evidence of any organic solvents or cigarette smoke. The breath samples were collected in specially constructed bags, which are provided along with the instrument. Exhaled breath H_2 was measured on a Model 12i Microlyser (Quintron Instrument Co., Milwaukee, WI, USA). The number of loose bowel motions and flatulence during the test were also documented.

Lactose tolerance test (LTT)

Following the above mentioned oral administration of 25 g lactose, capillary blood glucose levels were measured at 0, 60, and 120 minutes, by using a glucometer (Ascensia Contour, Bayer AG, Leverkusen, Germany). An increase of blood glucose by less than 1.1 mmol/l in conjunction with the development of abdominal symptoms was defined diagnostic for LI.[17]

Statistical analysis

Baseline data are reported as median (interquartile range, IQR), or numbers (percentages) as appropriate. Differences in baseline characteristics and between the 2 study groups were estimated using the Mann-Whitney U test for continuous variables and the χ^2 test for categorical variables. Values of exhaled breath hydrogen concentrations and blood glucose are presented as median (interquartile range, IQR). Differences of these values between different points in time were calculated using the paired sample Wilcoxon signed rank test. *P*-values of all outcomes were 2-sided; values less than 0.05 were considered to indicate statistical significance. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 22 (IBM Corporation, Armonk, NY).

Results

Of approximately 800 patients in the cohort of adult RTRs at the University Hospital Zürich, 22 individuals were identified with chronic diarrhea, 15 of which could be included in the present study (Figure 1). Four individuals were excluded due to primary LM in line with an expected frequency of primary LM in Switzerland of 20-40%[11] Seven of these 15 individuals had chronic symptomatic norovirus infection with diarrhea with the genotype G2.4 (noro group), and 8 patients had diarrhea in the absence of norovirus infection and served as control group. Baseline characteristics of the subjects in both groups are shown in Table 1, and laboratory data are summarized in Table 2. Cytomegalovirus high risk constellation (CMV donor/recipient serostatus D+/R-) was significantly more prevalent in the control group (p=0.013). However, the onset of diarrhea before study inclusion was significantly earlier in the noro group (p=0.038). Other variables, such as age, sex, mode and dose of immunosuppression, prevalence of diabetes mellitus, and laboratory values were comparable in both groups. At the moment of the testing, no patient was treated with antibiotics, and CMV PCR was negative in all patients. As part of the clinical routine diagnostic in transplanted patients with chronic diarrhea, 6 patients (85.7%) in the norovirus group had a colonoscopy. In all patients, the histology showed chronic inflammatory changes. CMV-colitis was specifically excluded with histology and immunohistochemistry of biopsy specimens. The final diagnosis of chronic diarrhea in those patients without evidence of norovirus infection was mycophenolate-associated colitis in 3 and unknown etiology or diabetes mellitus in 5 cases.

In the noro group, all patients had a positive LHBT. In the control group, only 1 patient (12.5%) had a positive test (Table 3). Accordingly, the increase of the median exhaled H_2 content

between baseline (7.0 (2.0, 11.0) ppm) and 120 minutes after lactose ingestion (35.5 (10.0, 66.2) ppm) in the noro group was significant (p=0.043) (Figure 2). By contrast, in the control group H_2 values only a minor, nonsignificant increase between baseline (4.0 (1.2, 6.0) ppm) and after lactose exposure was observed (9.0 (4.0, 16.2) ppm) (p=0.063) and all values remained below the threshold of 20ppm.

Analogously, LTT was positive in all patients in the noro group, whereas only the above mentioned patient in the control group had a positive test (Table 3). However, in both groups the increase of blood glucose between baseline and 60 min after lactose ingestion was significant (Figure 3), although the difference was more pronounced in the control group (baseline 5.3 (5.1, 6.6) mmol/l; after 60 min 7.9 (5.9, 8.5) mmol/l) (p=0.017) compared to the noro group (baseline 5.4 (4.8, 5.5) mmol/l; after 60 min 6.4 (5.7, 7.0) mmol/l) (p=0.046).

In all but 1 patient of the noro group, there were abdominal symptoms after lactose ingestion (diarrhea, n=2; bloating, n=3; combination of diarrhea and bloating, n=1). In the control group, no patient reported abdominal symptoms (Table 3). The patient in the control group with positive LHBT and LTT denied any symptoms after lactose ingestion.

Based on both tests, secondary LM was highly prevalent in the noro group compared to the control group with an odds ratio of 75.0 (95% CI 2.6, 2153), p=0.01. Likewise, for secondary LI (defined as LM with symptoms), the odds ratio was 73.7 (95% CI 2.6, 2120), p=0.01 (Table 3).

Discussion

Chronic norovirus infection is an emerging challenge in the immunocompromised host, in whom it may present as chronic diarrhea. The aim of the present study was to investigate

whether secondary LM can contribute to diarrhea in patients with chronic norovirus shedding. This is the first report showing a positive association of chronic norovirus infection in RTRs and secondary LM, suggesting a causative relationship between both entities. In addition, LI was highly prevalent, and diarrhea lasted substantially longer in RTRs with symptomatic chronic norovirus infection. Thus, secondary LM due to chronic norovirus infection could possibly be another cause of chronic diarrhea beside drug-induced diarrhea (eg mycophenolate) in immunosuppressed patients. Schorn et al found in their case series of RTRs with chronic norovirus infection, that the intensity of immunosuppression correlated with diarrheal symptoms but not with viral shedding.[6] Thus, immunosuppression dosage is maybe the most important risk factor for chronic norovirus infection. Therefore, we generally follow a stepwise approach with first reducing the dosage of mycophenolate because of the possibility of a coincident mycophenolate toxicity contributing to the chronic diarrhea, followed by reduction of the calcineurin-inhibitor dosage and attempt to taper/stop prednisone therapy. In our study, immunosuppressant dosage was similar in both groups in our study. However, lymphocyte counts were significantly lower in the noro group.

In general, endoscopy was performed for ongoing chronic diarrhea to rule out other conditions. However, colonoscopy was not an inclusion criterion in this study, although most of the patients had the procedure performed prior to study inclusion. Our standard procedure in RTR's with chronic diarrhea is CMV-PCR in blood, stool cultures for bacteria including Clostridium difficile and Clostridium toxin detection, PCR analysis of stool specimens for norovirus, and microscopy for parasites such as Microsporidium and Cryptosporidium. If the tests are negative, and the diarrhea persists, we first reduce the mycophenolate doses or change to enteric coated mycophenolic acid and try to reduce the cumulative immunosuppressive dosage according to the

immunological risk. If the diarrhea still persists, patients underwent a colonoscopy to look for other causes such as CMV colitis or mycophenolate toxicity.

LHBT is a standard diagnostic test for LM in clinical practice. However, 2 potential limitations should be mentioned: Small intestine bacterial overgrowth (SIBO) with lactose fermentation and H_2 production in the small intestine could potentially lead to a false positive LHBT. However, SIBO and LM can be distinguished, since the resulting H_2 peak will be early in the former (small bowel peak), but delayed and more prominent in the latter (colonic peak).[18] Furthermore, in a variable fraction of individuals (2-43%, <10% in most studies) the bowel flora does not produce H_2, leading to a false negative LHBT.[16]

Limitations of LTT include fluctuations of blood sugar levels for instance due to impaired glucose tolerance, diabetes or other influences.[19] The small increase in blood sugar levels required for a positive test will thus result in an inferior sensitivity and specificity of LTT, therefore this test is not recommended as a routine diagnostic test for LM.[10,20] However, since limitations of LHBT and LTT are largely nonoverlapping these tests can complement each other. In our study we found a perfect agreement of both tests regarding LM, arguing for the validity of our results.

Importantly, in the noro group but not in the control group, patients reported symptoms after lactose ingestion, suggesting that patients with chronic norovirus infection in fact suffer from LI. For most individuals with LM, a blinded challenge with 25 g of lactose does not result in any symptoms.[21] Therefore, symptoms after lactose ingestion in individuals with LM are likely due to the concomitant presence of visceral hypersensitivity, for instance due to irritable bowel syndrome (IBS). This was also suggested in a blinded controlled study where a challenge with 20

g lactose resulted in typical symptoms in 47% of patients with IBS but only in 22% of control patients.[22] Postinfectious IBS has been reported as a complication of viral gastroenteritis.[23] Our patients were not formally tested for the presence of IBS. However, our data suggest that visceral hypersensitivity can also complicate chronic norovirus infection.

Secondary LM has been reported in patients with intestinal inflammation due to other chronic inflammatory conditions including Crohn's disease.[24,25] In a rat model of mucositis, lactose digestion was severely reduced, along with downregulation of lactase mRNA and protein levels, while glucose absorption remained intact.[26] In this study, other intestinal disaccharidases were also downregulated, suggesting that lactase might be a marker for a more general derangement of digestive enzymes. Clearly, an isolated downregulation of lactase does not explain chronic diarrhea in norovirus infected patients since most individuals with primary LM are free of symptoms. Therefore, further mechanistic studies regarding the expression of lactase levels and other disaccharidases are clearly needed.

We are conscious that our study has several limitations. First, the sample size is small which is due to the low prevalence of symptomatic chronic norovirus infection. Secondly, since our study focused on RTRs the results might not be extrapolated to other immunocompromised disease states in which chronic norovirus infection is a relevant concern.[8] Thirdly, the investigators were not blinded for the results of norovirus PCR before lactose tolerance testing and no blinded placebo control was done as suggested by an NIH conference addressing LI.[27] Thus, placebo effects for the development of symptoms cannot be totally excluded. Finally, no additional tests regarding intestinal malabsorption were performed and our study does not provide a comprehensive information regarding all aspects of intestinal malfunction in norovirus infection.

Conclusion

This is the first study to show a positive association between chronic norovirus infection and secondary LM in RTRs. Future studies should address whether a lactose-reduced diet might be of therapeutic benefit.

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Figure 1. Study flow chart

LHBT, lactose H_2 breath test; LTT, lactose tolerance test

Figure 2. Lactose H_2 breath test

Exhaled H_2 content before and after ingestion of 25 g lactose

Figure 3. Lactose tolerance test

Serum glucose before and after ingestion of 25 g lactose

Figure 1

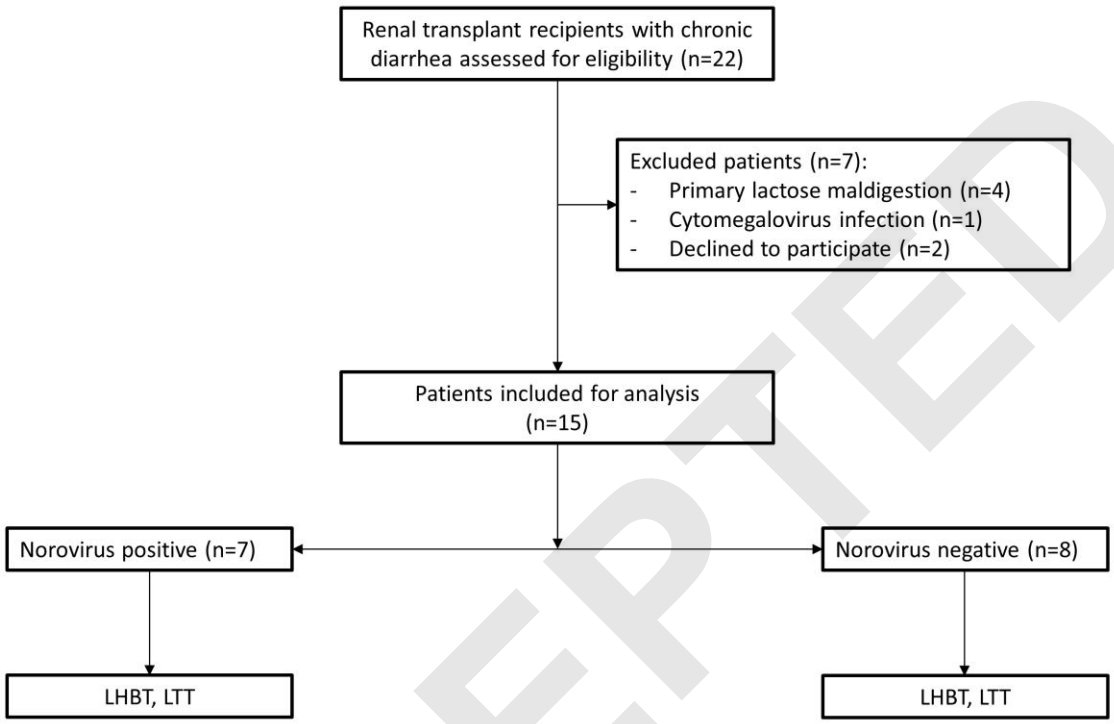

Figure 2

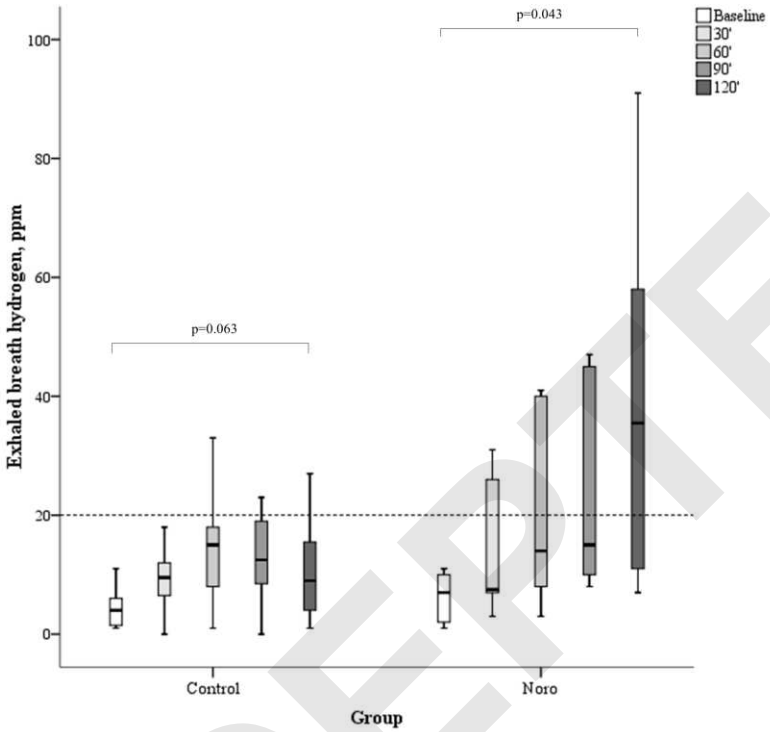

Figure 3

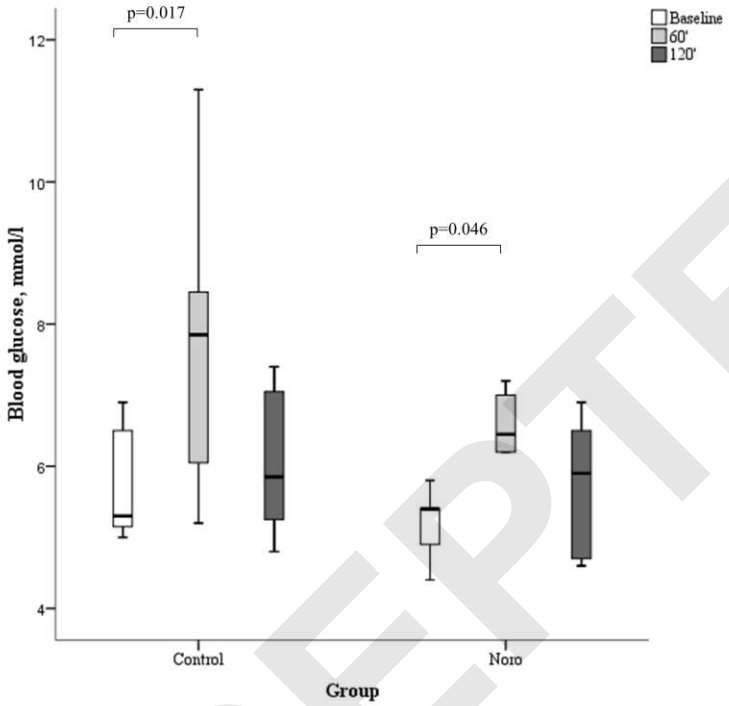

Table 1. Baseline characteristics

	Control group (n=8)	Noro group (n=7)	*p*-value
Age, years	52 (47, 60)	50 (34, 56)	0.46
Male sex	6 (75)	4 (57)	0.46
BMI, kg/m^2	24.8 (23.2, 26.5)	22.2 (17.0, 24.4)	0.09
Time since transplantation, years	2 (1-4)	6 (2-9)	0.23
RTx/RPTx	7/1	5/2	0.44
Immunosuppression, n (median daily dose; through level)			
- **Prednisone**	2 (5 mg)	1 (10 mg)	0.87
- **Azathioprine**	0	1 (150 mg)	0.69
- **Mycophenolate**	8 (1250 mg)	6 (1000 mg)	0.23
- **Ciclosporine**	1 (150 mg; 51 µg/l)	1 (90 mg; 50 µg/l)	0.78
- **Tacrolimus**	7 (4 mg; 7.3 µg/l)	6 (3.3 mg; 6.8 µg/l)	0.85
CMV risk constellation			
- **High (D+/R-)**	4 (50)	0 (0)	0.013
- **Intermediate (D+/R+ or D-/R+)**	2 (25)	7 (100)	
- **Low (D-/R-)**	2 (25)	0 (0)	
CMV PCR negative	8	7	0.46
Duration of diarrhea, weeks	13 (6-22)	35 (24-46)	0.038
Diabetes mellitus	3 (38)	2 (12)	0.31

Data are presented as median (interquartile range), or numbers (percent). BMI, body mass index; CMV, cytomegalovirus; PCR, polymerase chain reaction; RTx, renal transplantation; RPTx, combined renal-pancreatic transplantation

Table 2. Baseline laboratory values

	Control group (n=8)	**Noro group (n=7)**	***p*-value**
Hemoglobin, g/l	135 (118, 147)	122 (106, 133)	0.46
Leucocytes, G/l	6.1 (4.8, 6.9)	4.1 (4.0, 6.0)	0.12
Lymphocytes, G/l	1.42 (0.93-1.61)	1.00 (0.57-1.05)	0.041
Alanine transaminase, U/l	20 (14, 24)	22 (17, 37)	0.28
Alkaline phosphatase, U/l	56 (55, 81)	80 (55, 92)	0.71
Estimated glomerular filtration rate*, ml/min/1.73 m^2	48 (43, 58)	43 (40, 48)	0.40
Potassium, mmol/l	4.1 (4.0, 4.1)	4.4 (3.8, 4.8)	0.40
Sodium, mmol/l	140 (138, 140)	141 (139, 143)	0.28
Glucose, mmol/l	5.4 (4.8, 6.0)	5.1 (5.0, 5.5)	0.69
C-reactive protein, mg/l	0.9 (0.6, 1.5)	0.4 (0.3, 0.5)	0.054

Data are presented as median (interquartile range). * CKD-EPI equation.

Table 3. Test results of LHBT and LTT

	Noro group (n=7)	Control group (n=8)
LHBT / LTT positive	7 (100)*	1 (12.5)*
GI symptoms after lactose	6 (85.7)[#]	0 (0)[#]

Values are displayed in n (%). * p=0.001; [#] p=0.057.

GI, gastrointestinal; LHBT, lactose H_2 breath test; LTT, lactose tolerance test